Influence of soil-borne pathogens on seedling performance in declining *Quercus suber* forests: an experimental approach

Master thesis

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Declaration

I hereby declare that this master thesis is the product of my own work and that I did not use any other sources than the listed references. I did not receive any help or support from commercial consultants and I confirm that this master thesis has not been submitted as part of another examination process.

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Abstract

Soil-borne pathogens drive important problems of forest decline worldwide. In the Iberian Peninsula in particular, the exotic oomycete pathogen *Phytophthora cinnamomi* is driving the decline of cork oak (*Quercus suber*), one of the most abundant oak species in Mediterranean forests. This pathogen might not only influence community structure and composition by causing the death of adult cork oak trees, but also by limiting the establishment of new seedlings. This study aimed to experimentally analyse the effect of oomycete soil-borne pathogens on seedling performance of two different tree species (*Q. suber* and *Olea europaea*) that coexist in natural forests in southern Spain and show different susceptibility to *P. cinnamomi* attack. A greenhouse experiment was set up where seedlings grew on soils collected under trees of different species and health status in a declining cork oak forest. Half of the seedlings were treated with an oomycete-specific fungicide to investigate the impact of *P. cinnamomi* on aboveground and belowground plant performance. Seedlings were subjected to three competition levels (no competition, intraspecific competition and interspecific competition) to explore how pathogens might modify plant-plant interactions. *Q. suber* seedlings with fungicide showed faster growth and larger fine root biomass than control seedlings, especially on soils with high pathogen abundance sampled under defoliated cork oak trees in the field. The application of fungicide also altered the root morphology as well as the abundance of ecto- and arbuscular- mycorrhizal associations of the studied tree seedlings. Competiveness of oak and *Olea* seedlings was not strongly modified by oomycete pathogens. The results indicate that seedling performance of *Q. suber* in declining cork oak forests is strongly reduced by direct and indirect effects of *P. cinnamomi*. This recruitment limitation might cause difficulties to compensate for the death of adult trees, leading to a reduction in the abundance of this important tree species in Mediterranean regions.

Keywords

Phytophthora cinnamomi; fungicide; *Olea europaea*; competition; root morphology; mycorrhiza

Introduction

Tree decline and mortality is a relevant ecological problem affecting forest systems all over the word (Trumbore *et al.* 2015). Several global change drivers have been identified as potential causes, such as increasing drought frequency and severity (Millar & Stephenson 2015), or the spread of exotic pathogens (Gauthier *et al.* 2015). Increasing tree mortality rates could severely impair the capacity of forest ecosystems to act as carbon stores and to regulate the global climate system (Cramer *et al.* 2001). Therefore, sustainable regeneration is of particular importance to maintain such persistent ecosystems. The seedling stage, however, is recognised as the most vulnerable life stage of a tree and is thus less resistant to occurring stress events (Silvertown *et al.* 2001). In consequence of the raising appearance of tree decline, strong research efforts are currently directed towards understanding the causes and consequences of this phenomenon. The results provide a valuable scientific base for the design of mitigation and adaptation strategies.

Among tree species, those of the genus *Quercus* have been shown to be particularly vulnerable to problems of decline, while the main reason for this was ascribed to oomycete pathogens (Jung *et al.* 2000, Rizzo & Garbelotto 2003, Costa *et al.* 2010). A well-known example is the ‘sudden oak death’ in California (USA), caused by a soil-borne *Phytophthora* species (Rizzo & Garbelotto 2003, Meentemeyer *et al.* 2008). This emerging pathogen has caused a rapid decline of susceptible tree species and an increase in the relative dominance of coexisting species, changing the structure of the coastal forests of the region (Brown & Allen-Diaz 2009). In the Mediterranean basin, *Quercus suber* L. (cork oak) is one of the dominating evergreen tree species which also suffers from the attack of an oomycete pathogen, *Phytophthora cinnamomi* Rands (Costa *et al.* 2010, Camilo-Alves *et al.* 2013). This invasive soil-borne pathogen was identified as a cause for severe *Q. suber* decline in southern Europe at the end of the last century and since then has become a main ecological and social concern (Brasier 1992).

Oaks that are infected by *P. cinnamomi* show typical and clear visible signs of leaf necrosis, followed by crown defoliation and finally a sudden dieback of the whole plant (Aronson *et al.* 2009, Oßwald *et al.* 2014). From the infection it takes usually months up to several years until the first aboveground symptoms become visible (Oßwald *et al.* 2014), but once these appear, dieback can occur in adult trees within two years (Aronson *et al.* 2009). The symptoms caused by a *P. cinnamomi* infection are similar to those induced by drought stress (Luque *et al.* 1999) and for both stimuli even the same physiological signal pathways

are used within the plant (Oßwald *et al.* 2014). This makes it even more difficult to differentiate between the both possible sources for the detected damages. Several studies described yellowing and wilting of leaves, leaf drop-off, crown thinning and branch dieback as well as root necrosis and loss of feeder roots in oaks related to *P. cinnamomi* infection (Sánchez *et al.* 2002, Camilo-Alves *et al.* 2013). So far it is not known if and in which way this pathogen induces changes in the root morphology of susceptible plants. The expression of the root system in plants is related to their ecological strategy and can be altered in extent of their phenotypic plasticity according to environmental conditions (Grossman & Rice 2012). It has been reported that in response to pathogen infection cell walls are reinforced and more lignin is accumulated in roots (Pozo *et al.* 2002). Therefore, one might assume that such defence reactions are also reflected in the root morphology, e.g. by thicker roots with higher tissue density. Also the interaction with mycorrhizal fungi is an important aspect for plants that have to cope with root infesting pathogens (Azcón-Aguilar & Barea 1996, Pozo *et al.* 2002). However, such interactions are highly species dependent and only few studies have looked at the role of mycorrhiza in this context so far. Therefore it remains unconsidered how mycorrhizal symbiosis act on interactions between *Quercus* spp. and *P. cinnamomi*.

In natural systems soil-borne pathogens usually show heterogeneous spatial patterns in accordance with the distribution of potential host trees (Reinhart & Clay 2009). Zoospores of oomycete soil-borne pathogens are attracted by their host roots through root-secreted chemicals (Oßwald *et al.* 2014). These pathogens are reliant in their reproduction to susceptible living fine roots of a host and chlamydospores, as well as released zoospores accumulate close to the hosts root tissue. For example, in declining *Q. suber* forests of southern Spain the abundance of *P. cinnamomi* in the soil varied strongly at the scale of few meters depending on the species identity and health status of canopy trees (Gómez-Aparicio *et al.* 2012). The lowest *P. cinnamomi* abundance was found under tree species that do not act as hosts or in gaps that opened after the dead of cork oak trees, whereas the highest pathogen abundance was detected under clearly defoliated cork oaks. These differences in the spatial distribution of soil-borne pathogens might in turn influence new establishing plants and hence, also the offspring of the already infected individuals. According to that, the upcoming seedling generation of declining forest stands might be expected to be more affected on soils with high pathogen abundance than on soils with low pathogen abundance. Understanding the effects of soil pathogens on regeneration is crucial to make predictions of succession dynamics and long-term forest composition.

The effect of invasive soil-borne pathogens, such as *P. cinnamomi*, on regeneration patterns can not only act directly (i.e. by reducing performance of susceptible seedling species) but also indirectly by altering competitive interactions among coexisting species in the seedling/sapling bank. Host-specific pathogens are capable to act as selective force and change community compositions (Van Der Heijden *et al.* 2008, Hodge & Fitter 2013). When susceptible species are declining, they might be replaced by other unaffected species (Van der Putten & Peters 1997). Thus, the competitiveness of coexisting species is crucial for the composition of these species within a community. On the Iberian Peninsula *Q. suber* often coexists with the drought-resistant *Olea europaea* var. *sylvestris* Brotero (wild olive) forming open woodlands (Aronson *et al.* 2009). In contrast to *Q. suber, O. europaea* is known to be not or just barely affected by *P. cinnamomi* (Moralejo *et al.* 2009). *Q. suber* in general shows faster growth than *O. europaea* (Faria *et al.* 1998) and thus, can outcompete *O. europaea* on spots where required growth conditions are favourable for both species. However, it is not known so far how the effect of *P. cinnamomi* on *Q. suber* might alter the competitive balance among both species.

Even though cork oak decline in the Mediterranean basin is a relevant problem that has been observed already for several years, investigations on the impact of *P. cinnamomi* on the growth of *Q. suber* seedlings and on the role of mycorrhizal associations in this interaction are rather scarce. The few studies available used inoculations under controlled conditions to investigate the effects of *P. cinnamomi* on seedling performance (Cordier *et al.* 1996, Sánchez *et al.* 2002). However, these methods usually do not reflect the true pathogen abundances existing in the field. Additionally, natural nutrient conditions and microbial community are neglected in these cases. Another useful way to experimentally explore pathogen effects on seedlings is the use of natural soils in combination with metalaxyl-based fungicides. This substance is oomycete-specific and does not affect true fungi, just like plant beneficial mycorrhizal fungi (Schwinn & Staub 1995). Such fungicides have been successfully used in several studies to assess the role of pathogens as limiting factors of seedling establishment and growth (Reinhart & Clay 2009, Maron *et al.* 2013). Therefore, they appear as a promising way to explore the effects of *P. cinnamomi* on performance of coexisting tree seedlings in Mediterranean forest soils.

This study aimed to experimentally analyse the effect of oomycete soil-borne pathogens on seedling performance of two different tree species (*Q. suber* and *O. europaea*) that coexist in natural forests in southern Spain. In these forests *Quercus suber* is affected by severe decline driven by the soil-borne pathogen *P. cinnamomi*. A greenhouse experiment was set up where seedlings of *Q. suber* and *O. europaea* were grown on natural soils collected under adult trees of different species and health status. These soils differed not only in the abundance of *P. cinnamomi* (Gómez-Aparicio *et al.* 2012), but also in their chemical properties associated to the process of *Q. suber* decline (Ávila *et al. in press*). The effect of pathogens was assessed using an oomycete-specific fungicide applied to half of the seedlings. As plants that grow in competition often provide a more sensitive responses to different soil parameters (Bever 1994), seedlings were also grown under different competition treatments. Interacting effects of fungicide, soil type and competition were studied on both aboveground and belowground seedling traits, with an additional focus on the role of ectomycorrhiza (EM) and arbuscular mycorrhiza (AM) associations in pathogen-seedling interactions. In particular following hypotheses were proposed: (1) Performance of *Q. suber* seedlings is more limited by oomycete soil-borne pathogens than performance of *O. europaea* seedlings (2) The effect of pathogens on seedling performance varies among soil types according to their pathogen load, with the largest effect found in soils with high pathogen abundance (3) The effect of pathogens on seedling performance is modified by the competitive environment, with the largest effects found under strong competition. Pathogens are also expected to reduce the competitive effects of *Q. suber* (a species highly susceptible to *P. cinnamomi*) on *O. europaea* (a non-susceptible species), with potential implications for the coexistence of the two species under natural conditions. Furthermore, this study aimed to investigate the effect of oomycete soil-borne pathogens on the root morphology of affected *Q. suber* seedlings in order to provide information about the role of mycorrhizal symbioses in the interaction of oomycete pathogens and their host plants.

Material and Methods

Site description

The study site is located in Los Alcornocales Natural Park in southern Spain. The climate is sub-humid Mediterranean with an annual mean temperature of 15.7 °C and an annual mean precipitation of 620 mm, while 95% of all rainfall occurs from October to May. Soil is mainly clayish, acidic and poor in nutrients. The Los Alconocales Natural Park contains the largest protected cork oak forest in the world with an approx. size of 90,000 hectares (Aronson *et al.* 2009). It shelters numerous rare and endangered species and is of high ecological and economical value. In the dry lowlands of the park, *Q. suber* forms mixed open woodlands with *O. europaea*. The shrubby understory is diverse and rich in endemic taxa (Aronson *et al.* 2009). *Q. suber* trees in the study area show clear symptoms of decline driven by the exotic pathogen *P. cinnamomi*, which has been detected in with high quantities in the soil (Gómez-Aparicio *et al.* 2012). *O. europaea* in contrast seems to be unaffected by this decline.

Soil sampling and soil measurements

Soil sampling took place in January 2013. Soil was sampled under trees of four different categories: healthy *Q. suber* (without symptoms of crown defoliation), defoliated *Q. suber* (with crown defoliation > 50%), dead *Q. suber* (with leafless canopy) and *O. europaea* (soil types are hereafter referred as healthy, defoliated, dead and olive). 10 individuals of average size (i.e. 30-40 cm dbh) per tree category were selected with a minimum distance of 5 meters to adult trees of other categories. Soil was collected in four cardinal directions within 2 meters from the trunk of each tree. Samples of each individual tree were combined to generate one single soil sample per tree. Field soil samples were directly transported to the laboratory, where they were sieved through a 2-mm mesh to eliminate stones and large roots. Fine roots where cut into pieces and added to the sieved soil. All instruments were sterilized with household bleach (10% sodium hypochlorite) between usage for different soil types. Five randomly chosen soil samples per tree category were analysed in the laboratory for proportion of sand (with the Bouyoucos hydrometer method), total organic matter (by calcination at 540 °C), ammonium and nitrate (extracted with KCl 2M and determined by spectrophotometry), phosphate (using the Bray-Kurtz method) and calcium, magnesium and potassium (extracted with ammonium acetate 1M and determined by atomic emission spectroscopy).

Greenhouse experiment

Seeds of *Q. suber* and *O. europaea* were sampled in the study site in autumn 2012. Their surface was sterilized with household bleach (10% sodium hypochlorite) for 5 minutes (Dickie *et al.* 2002). Sound acorns were selected using the flotation method (Gribko & Jones 1995). Acorns were weighed and sown in sterile substrate in the greenhouse. It is known that *Olea* seeds have in general a very low probability of germination (Rey *et al.* 2004). Therefore, seeds of *Olea* were made to germinate in vitro. The seeds were put in tubes with RUGINI + 10 g/l sucrose medium (García 1999) and kept in a growth chamber (23 ± 1 °C, 16 h/day photoperiod) until transplantation. Initial seedling height was measured when they were transplanted to the experimental pots.

The experiment was set up in January 2013. Soil samples of each category were mixed to ensure homogenous conditions and then filled into 1 L pots. Half of all pots were treated with the oomycete specific fungicide Armetil 5 G (IQV Agro España, S.L.) containing 5% metalaxyl, whereas the other half of the pots did not receive any chemicals. Metalaxyl is known to affect *Phytophthora* spp. and *Pythium* spp. (Sukul & Spiteller 2000), but rather reduces their presence instead of entirely eradicating them (Schwinn & Staub 1995). It is referred here as a fungicide, even though the target species are not true fungi. Fungicide was applied once in a month consisting of 4 g fungicide per m², which corresponds to 0.45 g/pot. This dose was chosen according to the maximum non-detrimental amount for plants recommended by the manufacturer. Seedlings were planted in the pots according to three competition levels: without competition (one seedling per pot), with intraspecific competition (two seedlings of the same species per pot), and with interspecific competition (two seedlings of different species per pot). Overall, the experimental design included three entirely crossed factors: fungicide, soil type and competition. Each treatment combination consisted of 10 replicates resulting in 400 pots with 640 seedlings (Fig. 1). Plants grew in the greenhouse under controlled conditions (T_{max}: 27.3 ± 2.9 °C, T_{min}: 6.3 ± 4.8 °C, PAR ~ 300 µmol/m²s) and were watered twice a week up to the soils field capacity. Pots were rearranged randomly once a month within the greenhouse. During the experiment, only one oak seedling and seven olive seedlings died.

To test for potential side effects of the fungicide on seedling performance, an additional small experiment was set up. Therefore, 20 pots per species with pathogen free soil (2:1 mixture of peat and river sand) were prepared. Half of the pots were treated with fungicide, while the other half served as control without fungicide. Seeds were sown directly in the pots and germinated seedlings grew without competition.

soil type		healthy		defoliated		dead		olive	
fungicide		F	WF	F	WF	F	WF	F	WF
competition	without	QU OL	QU OL	QU OL	QU OL	QU OL	QU OL	QU OL	QU OL
	intraspecific	QU+QU OL+OL	QU+QU OL+OL	QU+QU OL+OL	QU+QU OL+OL	QU+QU OL+OL	QU+QU OL+OL	QU+QU OL+OL	QU+QU OL+OL
	interspecific	QU+OL	QU+OL	QU+OL	QU+OL	QU+OL	QU+OL	QU+OL	QU+OL

Fig. 1 Experimental design including three factors: soil type, fungicide and competition. Soil type consisted of four levels: healthy, defoliated, dead and olive soil. Fungicide consisted of two levels: with fungicide addition (**F**) and without fungicide (**WF**). Competition consisted of three levels: without, with intraspecific and with interspecific competition. Competition treatment in this figure represents the seedling(s) per pot (**QU**=*Quercus suber*, **OL**=*Olea europaea*). Each treatment combination consisted of 10 replicates.

Seedling measurements

Harvesting took place four months after transferring the seedlings to the experimental pots. As not all plants were harvested on the same day, harvesting date was recorded in order to calculate the growth duration for each seedling. All plants were divided into stem, leaves and root. Fresh samples were used to measure stem height, basal diameter, leaf number, fine root traits with the image software WinRhizo Version 2009c (Regent Instruments Canada Inc. 2009), and EM root colonization. Three root traits were selected that regulate species responses to different environmental conditions: specific root area (SRA, root area per unit of dry mass), specific root length (SRL, root length per unit of dry mass) and tissue mass density (TMDr, root dry mass per volume). SRA was highly correlated with SRL ($r = 0.873$, $p < 0.001$) and TMDr ($r = -0.821$, $p < 0.001$) and therefore, results of SRA are not shown. All plant parts were oven-dried at 75 °C for two days. Afterwards stem, leaves, fine roots, non-fine roots (⌀ > 2 mm) and for *Q. suber* also the tap root of each seedling were weighed separately. As aboveground biomass was highly correlated with stem biomass ($r = 0.964$, $p < 0.001$) and total leaf biomass ($r = 0.987$, $p < 0.001$), only the results for aboveground biomass

are given. Likewise, belowground biomass of cork oak was highly correlated with tap root biomass ($r = 0.975$, $p < 0.001$) and therefore, results of tap root biomass are not shown. The root mass fraction (RMF) was calculated as the belowground dry mass per unit of total plant dry mass. Average growth rate (cm/day) was calculated for each seedling by subtracting the initial seedling height from the final plant height and dividing by the growth duration.

The analysis of EM colonisation was assessed only for *Q. suber* seedlings, since *O. europaea* is a typical AM species (Kachkouch *et al.* 2012). Five cork oak seedlings per treatment combination were randomly chosen (n = 120 seedlings) and analysed using the gridline intersect method (Giovannetti & Mosse 1980). Three root replicates per seedling were surveyed using a stereo microscope Zeiss Stemi 2000-C with 2x to 3.5x magnification. For each replicate total number of fine roots, number of root tips without EM and number of root tips with EM that touched the gridlines were counted. Exclusively visually sound fine roots and root tips were considered in the observation. The EM colonisation (expressed as percentage) was calculated for each seedling by dividing the number of root tips with EM by the total sum of root tips. To evaluate the amount of living root tips compared to the total amount of fine roots of a plant, an index was calculated by dividing the total sum of root tips by the total number of fine roots that touched the gridlines. This index describes the proportion of root tips within the fine root system of a plant and is hereafter referred as the root tip index. It takes values from zero to one, with values close to zero representing few root tips and values close to one indicating many root tips.

For the analyses of AM exclusively seedlings on soil from defoliated trees were chosen, as this soil type appeared to have the highest pathogen abundance in the field (Gómez-Aparicio *et al.* 2012). Only seedlings from two of the three competition treatments (without competition and with intraspecific competition) were analysed. Five seedlings per species, fungicide and competition treatment (n = 40 seedlings) were randomly chosen. Dried fine roots were cleared and stained according to Vierheilig *et al.* (1998). Roots of *Q. suber* were cleared for 60 min and roots of *O. europaea* were cleared for 15 min in 10% KOH at 85 °C. The roots of *Q. suber* were additionally cleared in 30% H_2O_2 for 15 min at 24 °C. Afterwards all roots were rinsed in tap water and stained in 5% ink-vinegar solution (Pelikan 4001 royal blue, Hannover, Germany; with household vinegar containing 5% acetic acid) for 3 min at 85 °C. Subsequently, roots were rinsed again and decolourised for 24 h in tap water (acidified with a few drops of vinegar). For each sample 30 1-cm pieces of stained fine roots were arranged on microscope slides and fixed with 60% lactic acid. AM structures were identified with 40x magnification and quantified with 20x magnification using an Olympus Tokio FHA

microscope. Frequency of mycorrhiza in the root system (F%) and intensity of the mycorrhizal colonisation in the root system (M%) were calculated according to Trouvelot *et al.* (1986). These two variables were clearly correlated ($r = 0.856$, $p < 0.001$). Therefore, only results of F% are shown.

Statistical Analysis

All plant traits were analysed using Generalized Linear Models (GLMs), with fungicide, soil type and competition as fixed factors. Initial seedling height and growth duration were included as continuous covariates, except for the average growth rate, which was analysed without any covariate. GLMs were performed with normal error distribution for continuous variables. Before performing the analyses these data were checked for normal distribution and homoscedasticity and transformed if necessary to improve normality. The leaf number was analysed using a quasipoisson distribution. For EM colonisation, AM colonisation and the root tip index a quasibinomial distribution was used. Finally, RMF was analysed using a binomial distribution. Due to low mortality during the experiment, survival rate was not analysed. All analyses were conducted for both species separately. In contrast to the other variables, GLM of AM colonisation included the fixed factors fungicide and competition. For pots with intraspecific competition only one seedling was random chosen and its data used for the statistical analysis, in order to avoid pseudoreplication. Plant traits of the seedlings that grew on pathogen free soil were analysed with the fungicide treatment as the only fixed factor and acorn fresh weight (only in the case of *Q. suber*) as a covariate. Chemical soil properties were analysed using GLMs with soil type as fixed factor and a normal error distribution. Significant outcomes of the analyses were further examined using the Tukey's test to detect differences among groups of a factor. In case of a significant interaction between fungicide and another factor, multiple two-tailed t-tests with Bonferroni correction were applied with fungicide as explanatory factor. For all statistical analyses the statistic software R version 3.2.5 (The R Foundation for Statistical Computing 2016) and RStudio version 0.98.1103 (RStudio, Inc. 2013) were used, with the additional package multcomp version 1.4-5 (Hothorn *et al.* 2016).

Results

The fungicide treatment had significant effects on aboveground and belowground traits of *Q. suber*, but had no effects on *O. europaea* seedlings (Tab. 1, Tab. 2). Aboveground, *Q. suber* seedlings had 24.5% higher stem height and 38.7% higher average growth rate with fungicide than without fungicide. Belowground, *Q. suber* seedlings had 11.9% higher SRL with fungicide than without fungicide. The effect of fungicide varied among soil types for some variables (i.e. significant Fungicide × Soil type interactions; Tab. 1, Tab. 2). The effect of fungicide on leaf number, total plant biomass, fine root biomass and TMDr was particularly large in soils from defoliated *Q. suber* trees, followed by soils from healthy *Q. suber* trees (Fig. 2, Appendix 1, Appendix 2). The fungicide effects were not modified by the competitive environment for most variables (i.e. non-significant Fungicide × Competition interactions; Tab. 1, Tab. 2), with the exception of TMDr and EM colonization. Fungicide application had significant positive effects on TMDr and negative effects on EM colonization mainly under intra-specific competition (Appendix 3). The fungicide had no side effects on seedling growth, as shown by the fact that the fungicide treatment had no effects on above- or below-ground traits of *Q. suber* and *O. europaea* seedlings that grew on pathogen-free soil (Appendix 4).

Fig. 2 Effects of fungicide treatment and soil types on fine root biomass of *Q. suber* and *O. europaea*. Bars indicate mean values and arrows indicate standard errors. For the significant fungicide-soil type interaction for *Q. suber* (Tab. 2) symbols show effects of the fungicide respective to the soil type (n.s., $p > 0.1$; **.** , $p < 0.1$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). *O. europaea* is shown for comparison purposes.

Tab. 1 Result of the GLMs for the aboveground variables and total plant biomass with the covariates initial height and growth duration. Displayed values are F-values. Values in bold indicate significant p-values (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Species	Factor	Stem height	Stem diameter	Leaf number	Aboveground biomass	Total plant biomass	Average growth rate
Quercus	Initial height	**29.343 *****	**16.077 *****	**17.817 *****	**26.864 *****	**10.224 ****	-
	Growth duration	**98.549 *****	**27.552 *****	**75.725 *****	**73.955 *****	**27.356 *****	-
	Fungicide (F)	**11.546 *****	0.522	**10.305 ****	1.886	0.004	**18.377 *****
	Soil type (ST)	**7.301 *****	**14.442 *****	**4.887 ****	**18.489 *****	**12.479 *****	**4.373 ****
	Competition (C)	**6.583 ****	**6.970 ****	**8.277 *****	**13.551 *****	**6.267 *****	**10.785 *****
	F x ST	2.177	0.323	**3.406 ***	1.438	**2.857 ***	1.503
	F x C	0.674	0.258	1.394	0.058	1.692	1.096
	ST x C	1.195	1.047	1.423	1.970	0.786	0.837
	F x ST x C	0.723	0.238	0.347	1.422	0.894	0.468
Olea	Initial height	**12.835 *****	**17.231 *****	0.418	**8.532 ****	**6.437 ***	-
	Growth duration	**151.462 *****	**111.570 *****	**121.308 *****	**109.862 *****	**124.478 *****	-
	Fungicide (F)	0.006	2.371	1.121	1.822	3.159	0.005
	Soil type (ST)	**7.618 *****	**7.778 *****	**2.755 ***	**6.152 *****	**5.960 *****	**5.997 *****
	Competition (C)	**35.067 *****	**32.929 *****	**33.837 *****	**37.765 *****	**42.333 *****	**21.724 *****
	F x ST	1.133	0.161	1.204	1.542	1.971	1.052
	F x C	0.708	0.071	0.011	0.312	0.083	0.843
	ST x C	**2.633 ***	1.972	2.063	**3.271 ***	**3.340 ****	1.708
	F x ST x C	1.298	**2.346 ***	0.872	1.318	1.872	0.654

Tab. 2 Result of the GLMs for the belowground variables with the covariates initial height and growth duration. Displayed values are F-values and for RMF Deviance. Values in bold indicate significant p-values (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Species	Factor	Fine root biomass	Belowground biomass	SRL	TMDr	RMF	EM colonisation	Root tip index
Quercus	Initial height	**4.544 ***	**4.568 ***	1.143	0.002	0.108	0.234	0.626
	Growth duration	**28.240 *****	**13.401 *****	< 0.001	0.035	0.001	**22.173 *****	0.687
	Fungicide (F)	3.037	0.312	**7.325 ****	0.063	0.017	**7.350 ****	2.749
	Soil type (ST)	**5.407 ****	**9.632 *****	**4.174 ****	**6.466 *****	0.433	**2.864 ***	0.769
	Competition (C)	0.135	0.389	2.266	1.424	0.824	**6.398 ****	0.748
	F x ST	**2.816 ***	1.514	0.371	**3.444 ***	0.206	0.895	1.119
	F x C	0.618	0.487	3.078	**8.272 *****	0.161	**3.578 ***	1.235
	ST x C	1.916	1.216	0.497	**4.412 *****	0.474	**3.199 ****	1.743
	F x ST x C	1.738	1.007	0.839	1.432	0.868	1.359	0.853
Olea	Initial height	**4.846 ***	**4.548 ***	1.174	1.849	0.020	-	-
	Growth duration	**113.506 *****	**132.321 *****	0.260	2.148	0.047	-	-
	Fungicide (F)	3.707	2.510	1.237	1.729	0.012	-	-
	Soil type (ST)	**5.626 ****	**7.292 *****	2.208	0.041	0.052	-	-
	Competition (C)	**19.805 *****	**24.123 *****	**5.861 ****	0.606	1.147	-	-
	F x ST	1.656	0.875	0.544	1.075	0.141	-	-
	F x C	1.435	0.849	2.534	0.017	0.073	-	-
	ST x C	**3.406 ****	**3.102 ****	1.986	1.535	0.131	-	-
	F x ST x C	1.647	1.968	0.814	0.498	0.059	-	-

Soil type and competition had effects on growth of *Q. suber* seedlings independently of the fungicide treatment (Tab. 1, Tab. 2). Stem height, stem diameter, aboveground biomass, average growth rate, belowground biomass, TMDr, and EM colonization were generally lower in soil from healthy and defoliated cork oak trees than in soil from dead cork oak trees and wild olive (Fig. 3, Appendix 5). The analysis of the chemical properties of the different soil types showed that healthy and defoliated soils had lower concentrations of K^+ and NO_3^- than the other soils (Appendix 6). All aboveground parameters of cork oak seedlings were significantly reduced with intraspecific competition but were not affected by interspecific competition (Fig. 4).

Fig. 3 Effects of fungicide treatment and soil types on aboveground biomass of *Q. suber* and *O. europaea*. Bars indicate mean values and arrows indicate standard errors. For the significant soil type effect for *Q. suber* (Tab. 1) different letters show differences among the soil types. *O. europaea* is shown for comparison purposes.

O. europaea seedlings were clearly affected by interactive effects of the soil type and the competitive environment (Tab. 1, Tab. 2). Intraspecific and particularly interspecific competition strongly reduced aboveground and fine root biomass of *O. europaea* in soil from defoliated cork oaks and from olive trees, but had weaker or negligible effects in soils from healthy and dead cork oak trees (Appendix 7, Appendix 8). The differences in *O. europaea* performance among soil types followed a similar pattern to that of phosphorous content (Appendix 6). The lowest PO_4^{3-} content was found in olive soil and the highest in soil from healthy cork oaks, with soils from defoliated and dead cork oaks showing intermediate values.

The intensity of AM colonisation in soils from defoliated cork oaks was 120% higher for *O. europaea* seedlings than for *Q. suber* seedlings. For *O. europaea* the AM colonisation was significantly influenced by the fungicide treatment in interaction with the competition treatment (Appendix 9). The application of fungicide increased the AM colonization of *O. europaea* seedling roots when they grew with intraspecific competition, but had no effects when they grew without competition (Appendix 10).

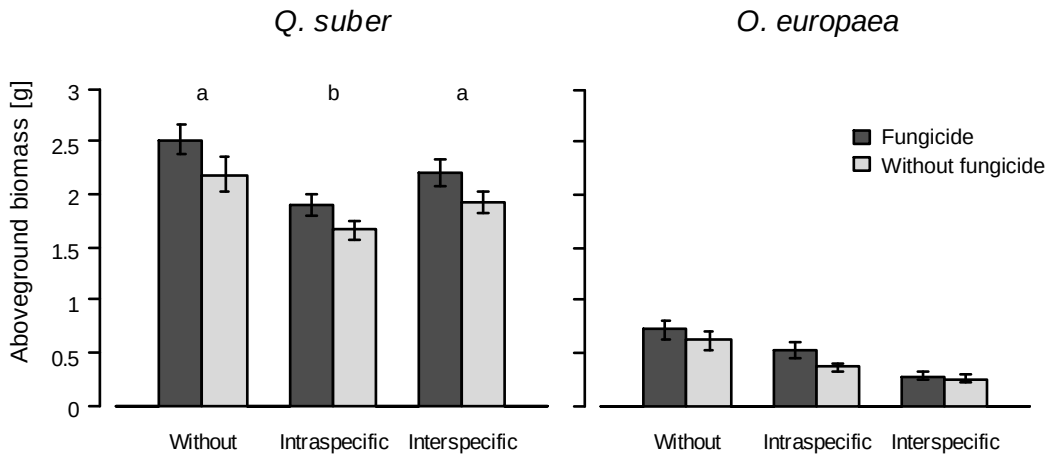

Fig. 4 Effects of fungicide and competition treatment on aboveground biomass of *Q. suber* and *O. europaea*. Bars indicate mean values and arrows indicate standard errors. For the significant competition effect for *Q. suber* (Tab. 1) different letters show differences among the competition treatments. *O. europaea* is shown for comparison purposes.

Discussion

Soil-borne pathogens limit seedling performance of Q. suber

The significant response of *Q. suber* to the applied fungicide treatment in this study support the hypothesis (1) that seedlings of this species are severely affected by oomycete pathogens which are abundant in natural soils. Likewise, the assumption is confirmed that *O. europaea* in contrast stays unaffected by these soil-borne pathogens. As the fungicide itself did not have any effect on the seedlings that grew on pathogen free soil, potential side effects of the fungicide can be falsified and the detected results can be ascribed purely to the target oomycete species of this fungicide, in this soils primary *P. cinnamomi*. These findings go along with previous studies that described a negative effect of *P. cinnamomi* infection on growth of oak seedlings under controlled conditions (Luque *et al.* 1999, Maurel *et al.* 2001). However, these studies used pathogen inoculations, which neither reflect the true pathogen abundance or the physico-chemical environment of natural forest soils. The present study in contrast used soils from a natural affected forest stand, making it more reliable to refer the results to field conditions. Thus, it can be expected that the natural regeneration of *Q. suber* is strongly affected by *P. cinnamomi*, whereas the regeneration of *O. europaea* remains unaffected by the direct effects of this pathogen. Other studies have demonstrated that *O. europaea* is also influenced by soil-borne oomycetes in Mediterranean regions (Sánchez-Hernández *et al.* 1997, González *et al.* 2015). However, these pathogen species seem not to be present in the studied soils, as fungicide did not have any effects on growth traits of *O. europaea*. Even if *P. cinnamomi* is referred as a generalist pathogen (Bergot *et al.* 2004), it does not infect *O. europaea* but *Q. suber* instead, with remarkable negative consequences on its seedling performance.

Several studies that have looked at the effect of oomycete pathogens on tree seedlings identified a significant negative effect on seedling survival (Reinhart & Clay 2009, Gripenberg *et al.* 2014). Also in the cork oak forest of study, a considerably seedling mortality associated to *P. cinnamomi* abundance was recorded under natural conditions (Gómez-Aparicio *et al.* 2012). However, in this experimental study no evidence for negative effects of pathogens on seedling survival was found. The most likely reason for this result might be that seedlings in this experiment were watered frequently and did not suffer from water shortage, which is the main factor driving mortality of *Q. suber* seedlings under natural conditions (Gómez-Aparicio *et al.* 2008). The strong reduction of fine root biomass caused by

oomycete pathogens in *Q. suber* seedlings (and consequently their lower water absorption capacity) might however be expected to result in a larger probability of seedling death under the natural conditions of summer drought that characterize Mediterranean forests.

Performance of Q. suber seedlings varies among soil types differing in pathogen abundance and chemical properties

The results of this study support the hypothesis (2) that the effect of soil-borne pathogens on *Q. suber* seedling performance varies among soil types according to their pathogen load, with the largest effect being found in soils with high pathogen abundance. Thus, the strongest fungicide effects were found in soil sampled under defoliated trees, where abundance of *P. cinnamomi* was highest in the field (Gómez-Aparicio *et al.* 2012). A similar pattern was observed in soil sampled under healthy trees, which is also characterised by large pathogen abundance. The lower fine root biomass of *Q. suber* seedlings growing in defoliated soil without fungicide could be a consequence of root necrosis and therefore is a clear indication of pathogen infection (Sánchez *et al.* 2002). These effects can be highly detrimental for seedlings, especially in dry Mediterranean climate, as fine roots are most crucial for water uptake (Eissenstat 1992). The reduced leaf number of *Q. suber* seedlings that grew on defoliated soil without fungicide might be also seen as a consequence of infection with oomycete pathogens, as this is a commonly described symptom in diseases caused by *Phytophthora* (Oßwald *et al.* 2014). Finally, the effects of pathogen infection are reflected in the reduced overall biomass of affected cork oak seedlings without fungicide mainly on soil derived from defoliated trees.

The results suggest that due to the severe impact of oomycete soil-borne pathogens in soils from defoliated cork oaks, establishment of *Q. suber* seedlings under natural conditions might by lower under these trees than under non-defoliated conspecific trees or under non-susceptible tree species. The beneficial effect of lower pathogen abundance under dead cork oak trees in contrast might be counterbalanced by the detrimental effect of a missing canopy shelter (Caldeira *et al.* 2014). In any case, the spatial patterns of soil-borne pathogens which go along with the health status of affected trees in a declining forest stand are also reflected in the seedling performance of the affected species.

Additionally to the pathogen abundance, other soil factors appeared to have an effect on seedling performance of *Q. suber*, as these seedlings performed differently on the different soil types independent of the fungicide treatment. Particularly, aboveground parameters were

reduced on soils from healthy and defoliated cork oak trees. Previous studies have shown that pathogen-driven decline of *Q. suber* has relevant effects on soil chemical properties, with soil fertility being reduced under defoliated and dead trees (Ávila *et al. in press*). This means in turn that seedling establishment of *Q. suber* is not only directly affected by soil-borne pathogens, but also indirectly affected by the modifications of soil chemical properties caused by the decline of infected adult trees.

Impact of competition according to pathogen infection

The competitive environment did not modify the effects of pathogens on seedling performance, contrary to the third hypothesis of the study. Similar positive effects of fungicide on performance of *Q. suber* seedlings were found under the three competition treatments. Also contrary to the third hypothesis, *Q. suber* was a stronger competitor than *O. europaea* regardless of the soil type and its pathogen abundance. Even without fungicide on defoliated soil, where pathogen abundance appeared to be highest, *Q. suber* showed more than twice as fast growth than *O. europaea*, which was significantly affected by interspecific competition. These results reveal that reduced seedling performance of *Q. suber* as a consequence of pathogen infection is not additive with effects caused by competition and therefore, does not change the natural occurring competition pattern. Studies about the competition of *Q. suber* and *O. europaea* are commonly rare. However, in this experiment *Q. suber* showed rapid growth resulting in superior competitive strength over slow growing *O. europaea* and strong intraspecific competition with conspecific oak seedlings. These competition patterns are typical for strong competitors and highly important to maintain the coexistence of both species (Connell 1983). Even if performance of *Q. suber* is clearly affected by soil-borne pathogens, its competitive ability still had a negative effect on *O. europaea*, indicating that the species coexistence of young *Q. suber* and *O. europaea* seedlings stays unaffected by soil-borne pathogens present in declining *Q. suber* forests.

The role of root morphology and mycorrhizal colonisation in plant-pathogen interactions

Root morphological traits provide valuable information to assess ecological strategies of plants (Westoby & Wright 2006). As far as known, this study is the first one that provides information about the effect of infection with oomycete soil-borne pathogens on plant roots morphology. The higher SRL and TMDr of cork oaks treated with fungicide indicated that

oomycete pathogens clearly affect root morphology of *Q. suber*, especially on defoliated soil which show very high abundance of these pathogens. According to Collins *et al.* (2016) high SRL and low tissue density are related with rapid resource acquisition, but also with low defence ability. Thus, the altered root morphology of affected *Q. suber* seedlings might be aimed to achieve a better defence against soil-borne pathogens.

Several studies proved the beneficial effect of mycorrhizal fungi on plants when they have to cope with soil-borne pathogens (Marx 1973, Azcón-Aguilar & Barea 1996). On the one hand they provide water and nutrients for the plants that are essential to increase their defence mechanisms. On the other hand EM build up a protective layer around the root tips, so that pathogens like *P. cinnamomi* cannot penetrate these tips unhindered (Marx 1973), while AM often induce resistance against pathogens (Cordier *et al.* 1998, Pozo *et al.* 2002). In this study *Q. suber* developed considerably less AM symbioses than *O. europaea*, but both species did not compete for AM fungi. Also the fungicide did not show a clear effect on AM colonisation, probably because AM symbioses are not restricted to the root tips and therefore they do not compete with the pathogens for the same host tissue.

Q. suber seedlings of all treatments invested in EM symbiosis. However, the percentage of EM colonization was lower in seedlings treated with fungicide than in control seedlings, although only under intraspecific competition. The TMDr of the same seedlings expressed exactly the opposite pattern. One possible explanation for these results is that oak seedlings without fungicide but with intraspecific competition showed lower performance than equivalent seedlings with fungicide or without competition and therefore they might try to compensate this deficit with increased mycorrhizal symbiosis. Another explanation might be that EM fungi are present with limited abundance in the soils and oak seedlings that grow together in intraspecific competition with fungicide compete for EM symbiosis, as both seedlings together build up more fine roots than one single seedling or two seedlings that are affected by soil-borne pathogens. However, this would suggest that EM colonisation has a rather indirect effect on the interaction between oomycete pathogens and *Q. suber* seedlings. Even though mycorrhizal symbiosis generally has a positive effect on plant performance, for declining *Q. suber* stands in southern Spain a net negative effect of soil microbial community on *Q. suber* seedlings has been found (Domínguez-Begines *et al.* 2014), indicating that the negative effect on these plants caused by soil-borne pathogens dominates over the positive effect produced by mutualistic EM symbioses.

Conclusions

This study provides important information about the effect of soil-borne pathogens on seedling performance of coexisting species in declining cork oak forests of southern Spain. The results shown here demonstrate that seedlings of *Q. suber* are strongly limited in their aboveground and belowground growth by invasive oomycete pathogens. Especially the high pathogen abundance under infected conspecific trees acts highly detrimental on *Q. suber* seedlings, indicating that establishment might be reduced under or close to these trees under natural conditions (Gómez-Aparicio *et al.* 2012). Additionally, the given results support that changes in abiotic conditions related to tree decline can influence the upcoming seedling generation (Ávila *et al. in press*). Invasive soil-borne pathogens, such as *P. cinnamomi*, might act as a selective force in infected forest, as the susceptibility to these pathogens strongly differs among coexisting tree species. However, the results of this study showed that *Q. suber* can grow faster than non-susceptible co-occurring tree species like *O. europaea*, both in the presence and absence of pathogens. Therefore, the natural regeneration of affected species might be reduced but the composition of coexisting tree species in declining *Q. suber* forests is not altered by oomycete soil-borne pathogens. In the long term, the recruitment limitation imposed by *P. cinnamomi* on *Q. suber* might cause difficulties to compensate for the death of adult trees, leading to a reduction in the abundance of this important tree species in Mediterranean regions.

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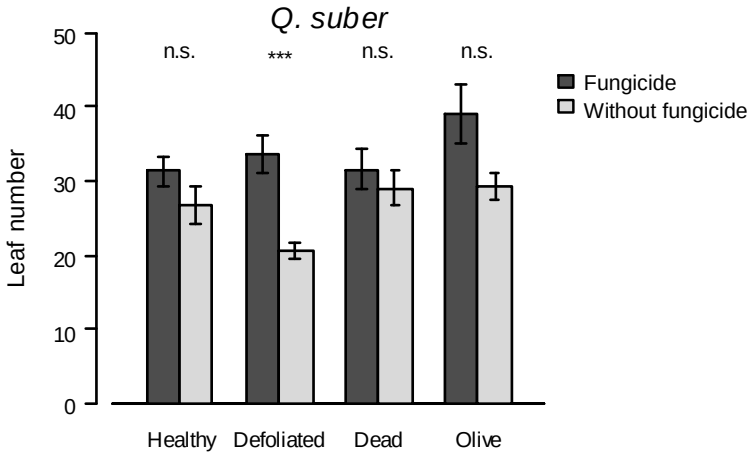
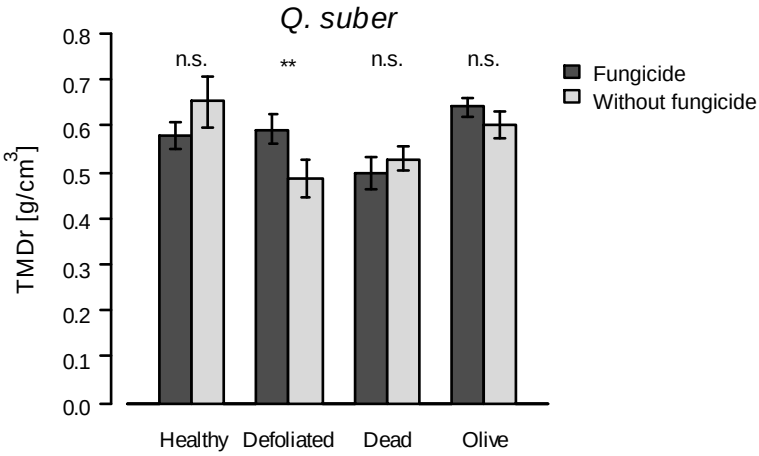
Appendix

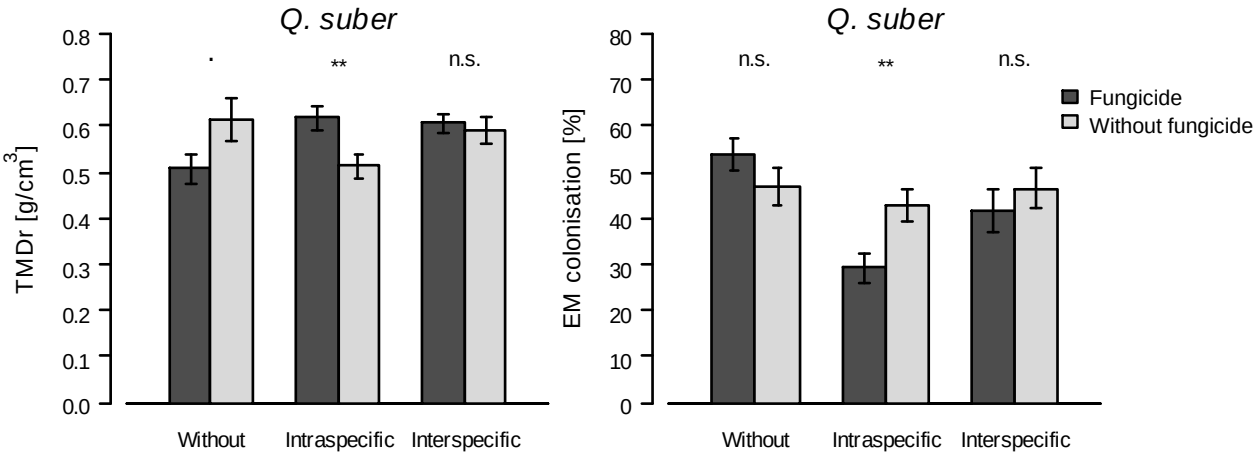
Appendix 1 Effects of fungicide treatment and soil types on leaf number of *Q. suber*. Bars indicate mean values and arrows indicate standard errors. For the significant fungicide-soil type interaction (Tab. 1) symbols show effects of the fungicide respective to the soil type (n.s., $p > 0.1$; **.** , $p < 0.1$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).



Appendix 2 Effects of fungicide treatment and soil types on TMDr of *Q. suber*. Bars indicate mean values and arrows indicate standard errors. For the significant fungicide-soil type interaction (Tab. 2) symbols show effects of the fungicide respective to the soil type (n.s., $p > 0.1$; **.** , $p < 0.1$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).



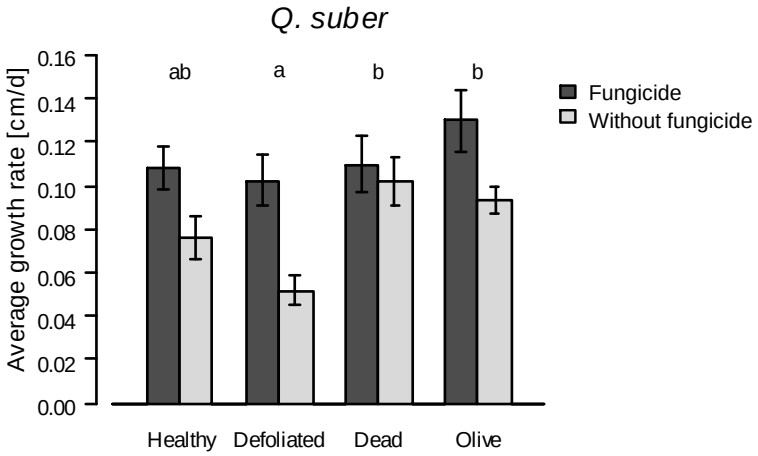
Appendix 3 Effects of fungicide and competition treatment on TMDr (left) and EM colonisation (right) of *Q. suber*. Bars indicate mean values and arrows indicate standard errors. For the significant fungicide-soil type interactions (Tab. 2) symbols show effects of the fungicide respective to the competition treatment (n.s., $p > 0.1$; **.** , $p < 0.1$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).



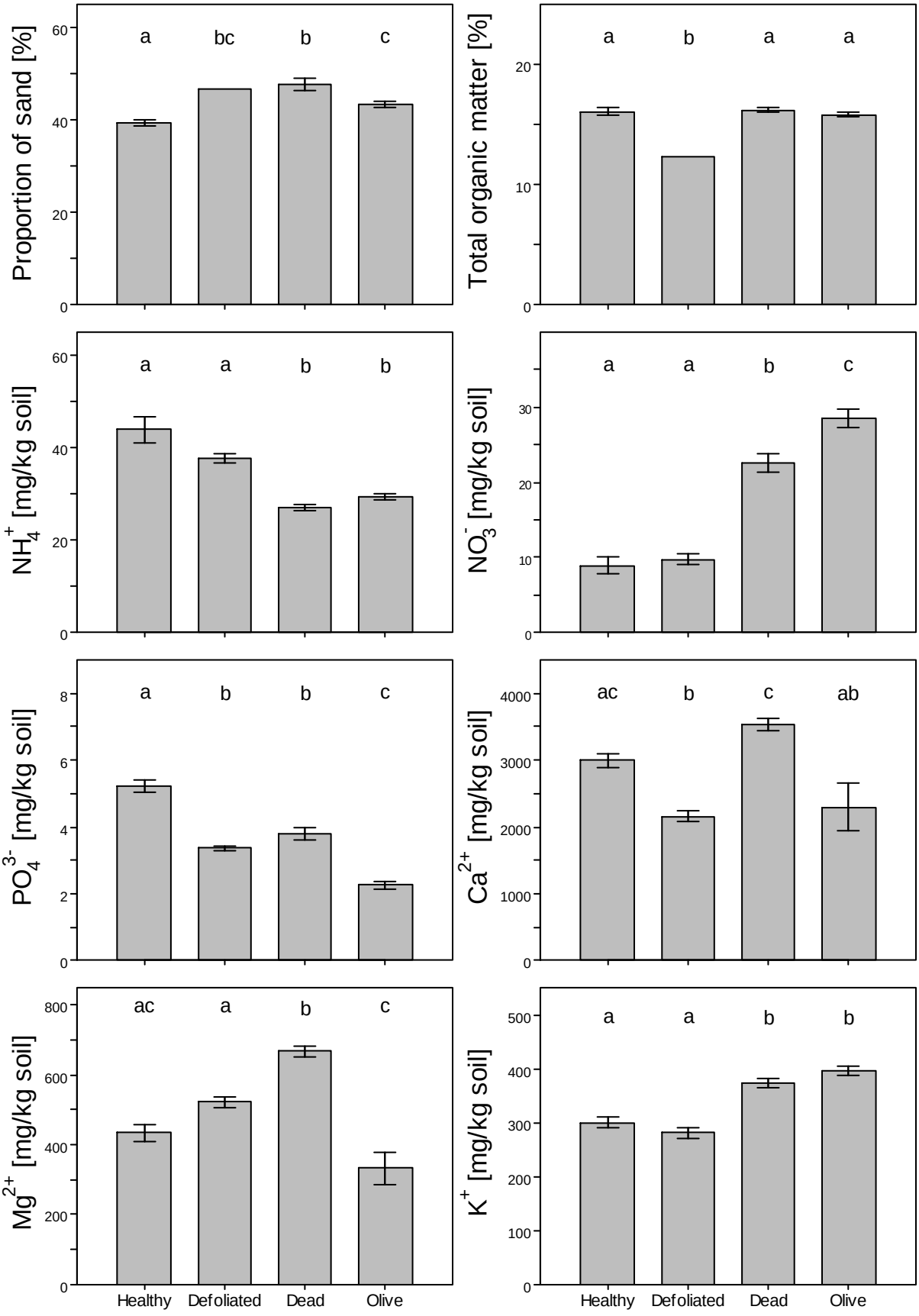
Appendix 4 Effect of the fungicide on both species on pathogen free soil with acorn fresh weight as covariate for *Q. suber*. Displayed values are F-values, $p > 0.05$ for all values.

Species	Factor	Leaf number	Stem length	Aboveground biomass	Belowground biomass
Quercus	Acorn fresh weight	**5.334 ***	3.115	**9.896 ****	**77.935 *****
	Fungicide	0.135	0.119	0.021	0.187
Olea	Fungicide	0.791	0.125	0.005	0.648

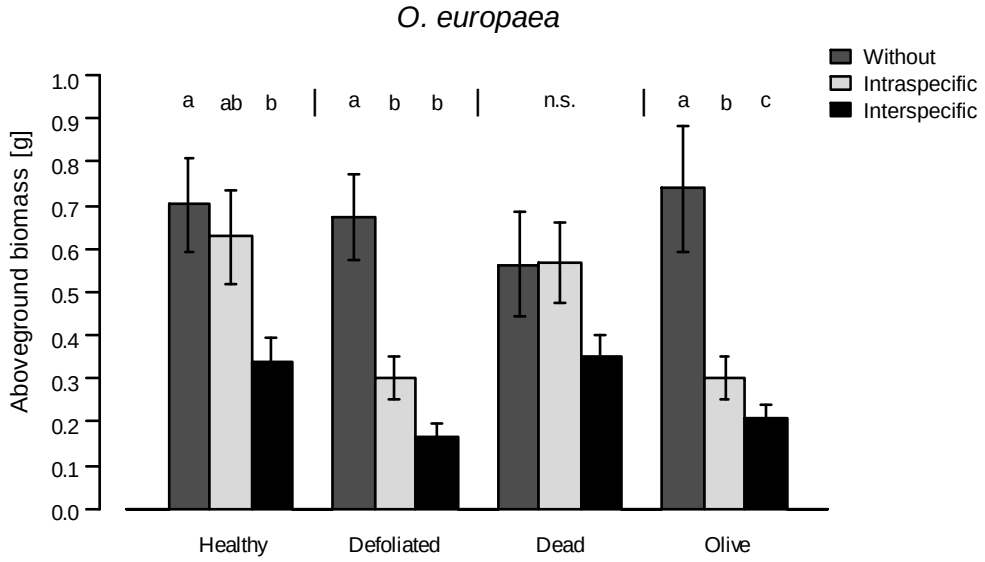
Appendix 5 Effects of fungicide treatment and soil types on average growth rate of *Q. suber*. Bars indicate mean values and arrows indicate standard errors. For the significant soil type effect (Tab. 1) different letters show differences among the soil types.



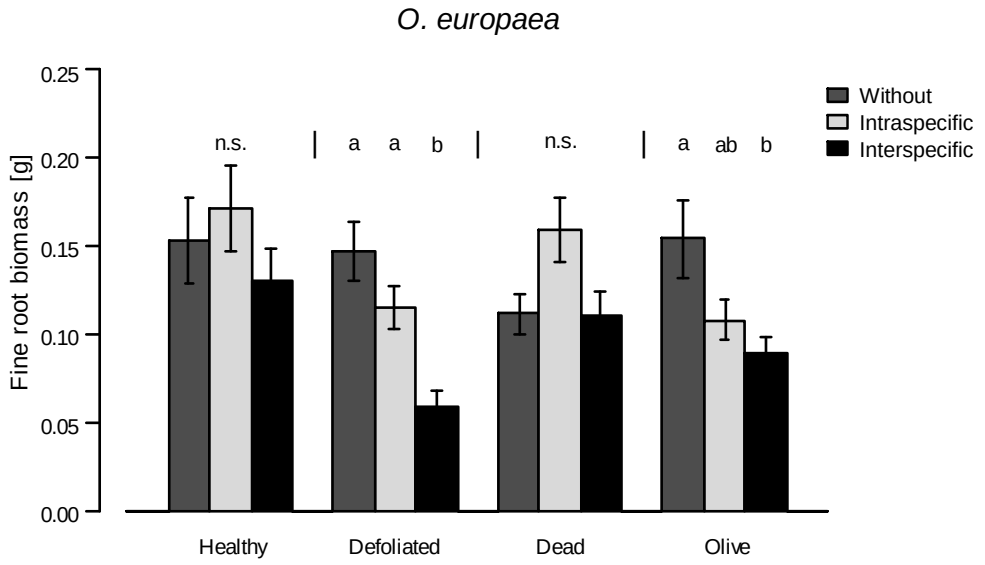
Appendix 6 Results of the tested soil properties. Bars indicate mean values and arrows indicate standard errors. Different letters show differences among the soil types.



Appendix 7 Effects of competition treatment and soil type on aboveground biomass of *O. europaea*. Bars indicate mean values and arrows indicate standard errors. For significant interactions of both factors different letters show differences within a soil type. Symbols show non-significant (n.s.) interactions.



Appendix 8 Effects of competition treatment and soil type on fine root biomass of *O. europaea*. Bars indicate mean values and arrows indicate standard errors. For significant interactions of both factors different letters show differences within a soil type. Symbols show non-significant (n.s.) interactions.



Appendix 9 Result of the GLM for AM colonisation with initial height and growth duration as covariates. Displayed values are F-values. Values in bold indicate significant p-values (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Species	Factor	Frequency of AM (F%)
Quercus	Initial height	1.489
	Growth duration	2.601
	Fungicide (F)	1.402
	Competition (C)	1.795
	F × C	0.325
Olea	Initial height	0.790
	Growth duration	0.735
	Fungicide (F)	0.128
	Competition (C)	0.695
	F × C	**6.901 ***

Appendix 10 Effects of competition treatment on Frequency of AM of *O. europaea*. Bars indicate mean values and arrows indicate standard errors. For the significant fungicide-competition interaction (Appendix 9) symbols show effects of the fungicide respective to the competition treatment (n.s., $p > 0.1$; **.** , $p < 0.1$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).